Review

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α-Synuclein Pathology in Synucleinopathies: Mechanisms, Biomarkers, and Therapeutic Challenges

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Abstract: Parkinson's disease and related synucleinopathies, including dementia with Lewy bodies and multiple system atrophy, are characterized by the pathological aggregation of the α-synuclein (aSyn) protein in neuronal and glial cells, leading to cellular dysfunction and neurodegeneration. This review synthesizes knowledge on aSyn biology, including its structure, aggregation mechanisms, cellular interactions, and systemic influences. We highlight the structural diversity of aSyn aggregates, ranging from oligomers to fibrils, their strain-like properties, and their prion-like propagation. While the role of prion-like mechanisms in disease progression remains a topic of ongoing debate, these processes may contribute to the clinical heterogeneity of synucleinopathies. Dysregulation of protein clearance pathways, including chaperone-mediated autophagy and the ubiquitin-proteasome system, exacerbates aSyn accumulation, while post-translational modifications influence its toxicity and aggregation propensity. Emerging evidence suggests that immune responses and gut microbiome alterations are key modulators of aSyn pathology, linking peripheral processes—particularly intestinal origin—to central neurodegeneration. Advances in biomarker development, such as cerebrospinal fluid assays, post-translationally modified aSyn, and real-time quaking-induced conversion technology, hold promise for early diagnosis and disease monitoring. Furthermore, positron emission tomography imaging and conformation-specific antibodies offer innovative tools for visualizing and targeting aSyn pathology *in vivo*. Despite significant progress, challenges remain in accurately modelling human synucleinopathies, as existing animal and cellular models capture only specific aspects of the disease. This review underscores the need for more reliable aSyn biomarkers to facilitate the development of effective treatments. Achieving this goal requires an interdisciplinary approach integrating genetic, epigenetic, and environmental insights.

Keywords: alpha-synuclein; synucleinopathies; Parkinson's disease; biomarkers; neurodegeneration; protein aggregation

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1. Introduction

Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are distinct neurodegenerative syndromes collectively known as synucleinopathies, as they are all marked by the presence of α-synuclein (aSyn) protein within the cytoplasm. These conditions differ based on the specific cellular compartments where aSyn accumulates. In PD and DLB, the protein is primarily found in Lewy bodies (LBs) within neurons, whereas in MSA, it is predominantly observed in glial cytoplasmic inclusions (GCIs)[1,2]. With numbers reflecting a significant public health impact, synucleinopathies have gained considerable attention in studying their molecular and pathological processes. Indeed, with approximately 2.5 million people in the United States suffering from some form of synucleinopathy[3] and more than 8.5 million globally[4], the urgency of improving early detection, treatment, and preventative strategies for PD and related synucleinopathies is evident.

aSyn was initially identified through an antibody recognising cholinergic vesicles from the torpedo electric organ[5]. This antibody highlighted aSyn expression at both the presynaptic level and the nuclear envelope, which led to the term "synuclein"[5]. Further studies characterized aSyn as a small protein composed of 140 amino acids. While its precise physiological role remains incompletely understood, it is believed to be involved in neurotransmitter release and facilitates transient synaptic vesicle fusion. The link between aSyn and neurodegeneration was first proposed after its presence was detected in amyloid plaques associated with Alzheimer's disease[5]. A pivotal breakthrough occurred in 1997 when Polymeropoulos and colleagues identified mutations in the *SNCA* gene in an Italian-American family with early-onset PD, which was confirmed to exhibit Lewy pathology upon autopsy[6]. That same year, Spillantini and colleagues demonstrated that LBs and Lewy neurites in the substantia nigra of patients with idiopathic PD and DLB exhibited strong aSyn immunoreactivity, firmly classifying PD and DLB as synucleinopathies[7]. A year later, independent research by Spillantini and Wakabayashi revealed the presence of aSyn in the GCIs of MSA patient samples [8,9].

Further key discoveries include findings that potentially different aSyn strains preferentially affect specific cell types and regions in the brain[10] and that aSyn aggregation patterns in cerebrospinal fluid differ between PD and MSA[11]. Misfolded aSyn can spread between neurons, forming cytoplasmic and/or nuclear inclusions in adjacent cells[12]. This observation has fueled suggestions that synucleinopathies share features with prion diseases, although no cases of direct human-to-human transmission have been documented. Notably, the relationship between clinical symptoms and pathological findings is not always straightforward, as aSyn aggregates have been detected in individuals without neurodegenerative disease, as well as in patients with other conditions. Additionally, indirect evidence from human studies suggests that protein aggregation may not always be inherently disease-causing[13].

In this regard, given the significant impact of synucleinopathies in patients, as well as the significant gaps regarding our knowledge of aSyn and its physio- and pathological mechanisms, this review explores the physiological structure, aggregation, clearance, and interactions of aSyn, alongside recent advances of biomarker research and mechanistic studies. We aim to highlight the key pathways underlying aSyn pathology and how their disruption contributes to different synucleinopathies. Furthermore, we describe the complexity of synucleinopathies underscoring the need for reliable biomarkers, with cerebrospinal fluid (CSF) aSyn levels, skin biopsy, and advanced seed amplification assays (SAA) like RT-QuiC and PMCA showing promise for early detection. We also highlight the animal and cellular models as crucial for studying aSyn pathology, although current models have limitations, with new approaches like CRISPR/Cas9 and patient-derived iPS cells offering potential improvements. We finalize by proposing novel potential treatments in line with current investigations.

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2. Physiological α-Synuclein: A Necessary Disordered Protein

2.1. Structure of α-Synuclein

aSyn is a small, highly dynamic protein composed of three distinct domains that collectively define its structural and functional properties (Figure 1)[14,15]. The N-terminal region contains four imperfect KTKEGV motif repeats, forming amphipathic helices essential for cellular membrane interactions[16]. The central hydrophobic domain, known as the non-amyloid component (NAC), is a key driver of aSyn aggregation and amyloid fibril formation. In contrast, the C-terminal region remains dynamically highly disordered [17,18] and is enriched with acidic and charged amino acids, which enhance solubility and regulate interactions with other proteins and ions[19]. These regions collectively enable aSyn's functional adaptability in physiological conditions and its pathogenic potential in disease states [20].

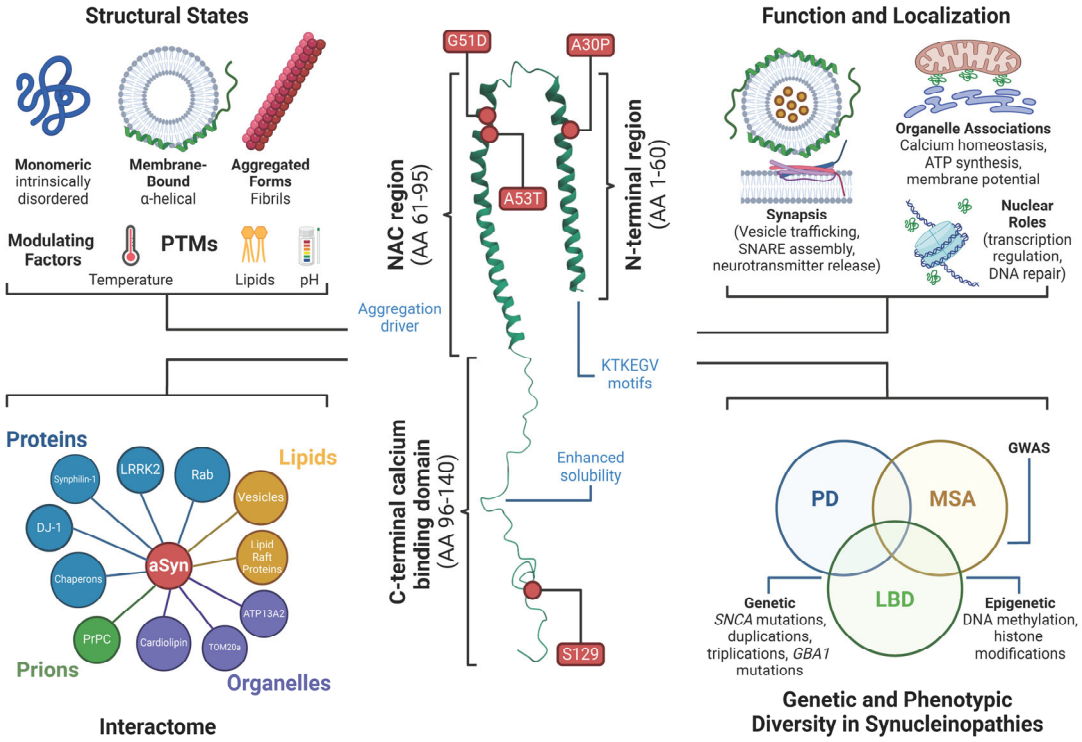

Figure 1. | Structural dynamics, molecular interactions, and pathological roles of alpha-synuclein. *Structural states of alpha-synuclein.* Alpha-synuclein exists in multiple conformations, including monomeric intrinsically disordered forms, membrane-bound α-helical structures, and aggregated fibrillar states, with its aggregation influenced by post-translational modifications (PTMs), pH, temperature, and lipid interactions. *Molecular interactome.* Alpha-synuclein interacts with a diverse range of molecular partners, including lipid membranes, SNARE proteins, cytoskeletal components, vesicular trafficking machinery, and organelles, modulating its physiological functions and pathological effects. *Physiological functions and localization.* Alpha-synuclein plays a key role in synaptic vesicle trafficking, neurotransmitter release, calcium homeostasis, ATP synthesis, and nuclear processes such as transcription regulation and DNA repair. *Genetic and phenotypic diversity in synucleinopathies.* Mutations, duplications, and triplications in *SNCA*, along with environmental and epigenetic factors, contribute to the aggregation and neurotoxicity of alpha-synuclein, underlying the pathogenesis of Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies.

Under normal conditions, aSyn is classified as an intrinsically disordered protein (IDP)[21,22], meaning it lacks a stable three-dimensional structure and primarily exists as an unstructured monomer. Its chemical shifts closely resemble a random coil[21,23], suggesting that, like other

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IDPs[24], its structure may be influenced by temperature and pH. However, upon interaction with cellular membranes or lipids, the N-terminal region adopts an alpha-helical conformation, a structural adaptation that promotes membrane curvature and stability[25–28]. Consistently, *in vitro* microfluidic studies that mimic the plasma membrane have shown that aSyn alters membrane topology by inducing pores, increasing membrane capacitance, and immobilizing lipids[29]. Lipids are crucial in modulating aSyn structure and function[25,26,30,31]. Beyond its monomeric and dimeric states, aSyn has been reported to form tetramers and higher-order quaternary structures[32]. However, although studies suggest these assemblies may be physiologically relevant—potentially regulating neurotransmitter release, glutamatergic receptor activity, and synaptic function—[33–37], their prevalence and functional significance remain debated[17,38,39].

2.2. Function and Subcellular Localization of α-Synuclein

aSyn was initially identified as a protein localized to both the synapse and the nucleus, reflecting its multifaceted functions and complex subcellular distribution (**Figure 1**)[5]. While its synaptic roles have been extensively studied—primarily due to their connection to neurodegeneration and LB pathology—its roles in the nucleus and other cellular compartments remain relatively underexplored, representing critical gaps in our understanding of its biology[7,40,41]. At the synapse, aSyn exists in a delicate equilibrium between soluble cytosolic and membrane-bound forms. It preferentially binds to lipid rafts enriched with unsaturated and polyunsaturated fatty acids and exhibits a strong affinity for highly curved membranes, such as synaptic vesicles[14,42]. However, the lipid composition modulates aSyn-induced docking of synaptic vesicles on the presynaptic membrane[43]. These properties allow aSyn to modulate essential synaptic processes, including vesicle trafficking, fusion, and neurotransmitter release. Through direct interactions with synaptobrevin, aSyn facilitates SNARE complex assembly, a process critical for vesicle fusion at the presynaptic terminal[39,44].

Beyond the synapse, aSyn is found in several organelles, exerting additional functions. Within mitochondria, aSyn interacts with the inner membrane through cardiolipin and the outer membrane via TOM20, a key protein involved in mitochondrial protein import[45,46]. aSyn also accumulates on the endoplasmic reticulum (ER) and Golgi membranes, where its overexpression disrupts ER-Golgi trafficking, induces ER stress and leads to Golgi fragmentation[47,48]. Additionally, aSyn is associated with mitochondria-associated membranes (MAMs), specialized regions that mediate ER-mitochondria communication and regulate calcium homeostasis. Dysfunction in MAMs has been implicated in synucleinopathies[49]. ASyn interacts with histones and DNA in the nucleus, influencing chromatin acetylation and modulating gene expression and DNA repair processes[50,51]. Although relatively understudied, these nuclear roles suggest broader regulatory functions that may contribute to neurodegenerative pathways.

Outside the nervous system, aSyn is highly expressed in peripheral tissues, particularly within red blood cells, platelets, and the gastrointestinal tract. Over 99% of aSyn in blood is localized to red blood cells, with smaller amounts found in platelets and plasma[52]. The functional roles of aSyn in peripheral tissues are poorly understood[40]. Notably, its detection in the gastrointestinal tract has attracted significant interest, particularly regarding its potential contribution to the gut-brain axis[53,54]. This observation supports the hypothesis that aSyn pathology could originate in peripheral sites and propagate to the central nervous system.

2.3. α-Synuclein Interactome Further Complexes Its Understanding

Extensive research has identified various aSyn interacting partners, including proteins, lipids, and nucleic acids. These interactions underscore aSyn's multifaceted roles in cellular physiology. Notable protein interactors include Synphilin-1[55], LRRK2[56,57], p25alpha[58], DJ-1[59], ATP13A2[60,61], and chaperones such as heat shock proteins[62,63]. aSyn also engages Rab family proteins to regulate vesicle trafficking[64,65], binds to heparan sulfate proteoglycans, and interacts

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with nuclear components like histones and DNA, further emphasizing its functional versatility[66,67].

In this regard, the expansive aSyn interactome complicates our understanding of its physiological functions. Interactions with lipid membranes further illustrate aSyn's role in cellular dynamics and disease. As previously described, the N-terminal domain is essential for binding to synaptic vesicles, axonal transport vesicles, and lipid rafts, processes integral to its physiological role in vesicle trafficking[42,68]. However, these interactions can also promote aggregation under pathological conditions. At the presynaptic terminal, aSyn interacts with proteins essential for neurotransmitter release, including Rab3, SNARE proteins, synapsin III, and vesicular monoamine transporter 2[44,69,70]. Moreover, aSyn regulates dopamine and serotonin transporters and modulates enzymatic activity, such as tyrosine hydroxylase function, further demonstrating its regulatory capacity in synaptic physiology[71,72].

Mitochondrial interactions are another crucial factor in aSyn pathology[73]. Although crucial for maintaining mitochondrial integrity, such interactions may become compromised in disease states, contributing to energy deficits and increased oxidative stress[46,74]. Furthermore, aggregated aSyn exacerbates mitochondrial dysfunction by directly impairing protein import and destabilizing membrane integrity[75]. These impairments amplify cellular stress, increase neuronal vulnerability and drive the progression of synucleinopathies[76].

However, despite significant progress, many aspects of aSyn interactome remain poorly defined[77]. Traditional approaches, such as co-immunoprecipitation and crosslinking, have yielded valuable insights but are limited by specificity and physiological relevance[78]. Emerging methodologies, including mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy, bimolecular fluorescence complementation, and proximity ligation assays, offer improved sensitivity and precision for detecting and characterizing aSyn interactions[79]. However, these approaches face challenges, including artefacts from overexpression systems and non-physiological conditions, underscoring the need for rigorous experimental validation[80]. Computational approaches that leverage molecular modelling and protein structure databases have demonstrated promise in predicting aSyn interactions[81]. Nonetheless, these predictions require stringent validation in models closely mimicking the human brain environment[82]. For greater translational relevance, *in vitro* systems must incorporate physiological expression levels, while *in vivo* models should recapitulate the tissue-specific contexts of synucleinopathies. High-resolution imaging and proteomics offer novel avenues for elucidating the dynamic nature of aSyn interactions across subcellular compartments[83]. Additionally, functional assays are needed to distinguish disease-promoting interactions from those that serve protective roles[84].

Deciphering aSyn's interactome is essential for understanding its biological roles and identifying potential therapeutic targets[40]. Targeting key interaction nodes within this network may provide strategic intervention points that preserve essential physiological functions while mitigating pathological effects. Integrating advanced experimental methodologies with computational tools holds significant potential for generating a more comprehensive understanding of aSyn's multifaceted roles in health and disease. These insights could ultimately facilitate the development of targeted therapies for synucleinopathies[40].

2.4. Mechanisms of α-Synuclein Clearance

Given the intrinsically disordered composition of a-Syn and its wide interactome and cellular distribution, the protein is susceptible to becoming pathological (**Figure 2**). Multiple cellular pathways regulate the aSyn clearance, including chaperone-mediated autophagy (CMA), macroautophagy, and the ubiquitin-proteasome system[85,86]. These mechanisms function to maintain aSyn homeostasis and prevent its pathological accumulation.

Macroautophagy plays a central role in the lysosomal degradation of oligomeric and aggregated aSyn species, as CMA is limited in handling large protein aggregates[87,88]. In contrast, the ubiquitin-proteasome system preferentially degrades monomeric aSyn for degradation[89].

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Interestingly, the proteasome can also partially process certain soluble oligomeric forms of aSyn[90]. Notably, mutant aSyn variants associated with familial PD, including H50Q, G51D, E46K, and A53E, are preferentially degraded by macroautophagy rather than CMA, suggesting distinct clearance pathways for mutant versus wild-type aSyn[91–93].

Beyond intracellular degradation, aSyn is actively secreted into the extracellular environment, where it can be taken up and degraded by neighbouring cells, including glial cells such as astrocytes and microglia, which demonstrate greater capacity for aSyn clearance than neurons[58,94]. However, internalized aSyn seeds can impair the endosomal-lysosomal machinery of recipient cells, promoting the aggregation of endogenous aSyn and propagating pathology[95]. PTMs further regulate aSyn turnover, influencing its aggregation propensity and degradation efficiency. For instance, ubiquitination, sumoylation, glycation, and phosphorylation modulate aSyn interactions with degradation pathways[96–100]. Its precise role in aSyn degradation remains unclear. Similarly, the effects of other PTMs, such as acetylation and nitration, require further investigation[76]. In this regard, animal models underscore the essential role of the autophagy-lysosomal pathway in regulating aSyn oligomers and aggregates[101–103]. In neurons, lysosomes serve as the primary site for internalized aSyn degradation, while proteases such as calpains [104,105] and metalloproteinases [106] contribute to intracellular and extracellular aSyn clearance[107]. However, whether these mechanisms remain efficient during ageing or become impaired in synucleinopathies remains uncertain.

Despite significant advances, key questions regarding aSyn clearance remain unresolved[108]. The relative contributions of different degradation pathways in processing specific aSyn species and the impact of ageing and disease on these pathways require further elucidation. Additionally, the roles of glial cells, particularly microglia and astrocytes, in aSyn clearance are insufficiently characterized despite their likely significance in disease progression [109,110]. Addressing these knowledge gaps necessitates advanced live-cell imaging techniques to examine the dynamics of aSyn degradation in real-time[79]. Tools for modulating and tracking PTMs could provide critical insights into their roles in aSyn clearance[111]. Furthermore, improved reporters and assays for assessing the activity of autophagy, proteasome, and lysosomal pathways will facilitate a more comprehensive understanding of their interactions with aSyn[112].

When the pathways responsible for clearing aSyn fail, the protein becomes increasingly vulnerable to misfolding and aggregation. As will be described later, this accumulation of pathological aSyn disrupts cellular homeostasis and contributes to neurotoxicity, ultimately driving the progression of synucleinopathies. A self-perpetuating protein aggregation cycle and neuronal dysfunction occur when these clearance mechanisms fail due to genetic mutations, environmental factors, or ageing-related decline.

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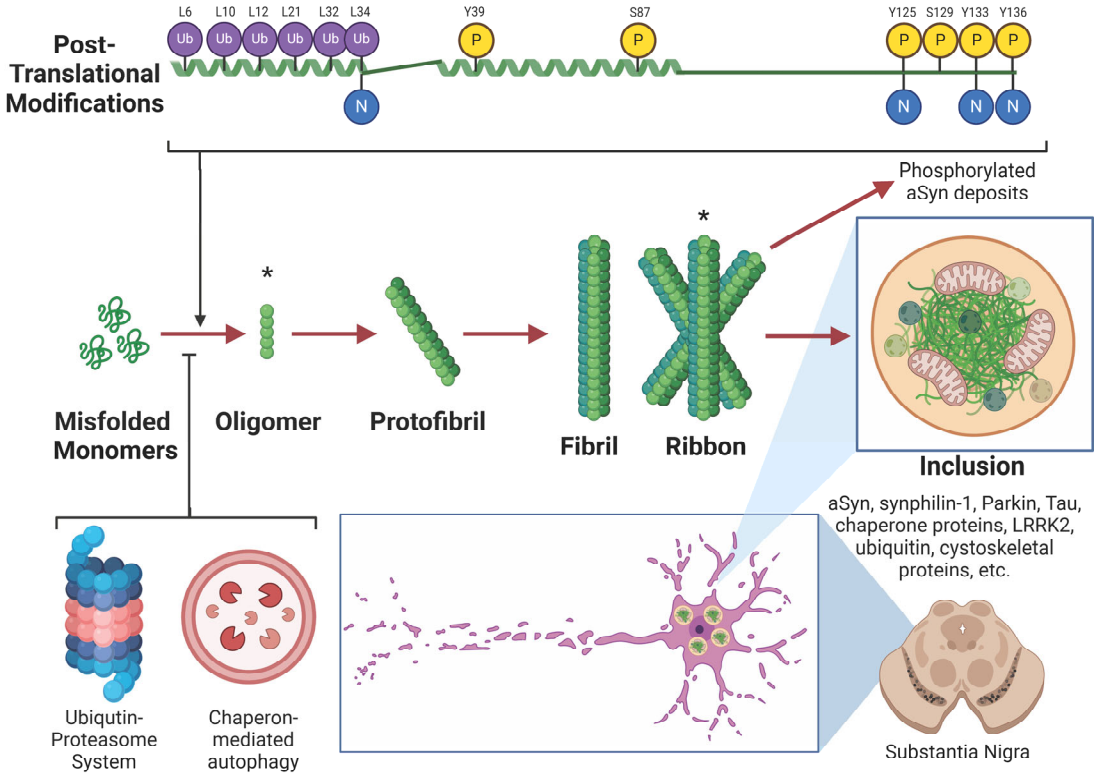

Figure 2. | Alpha-synuclein aggregation, post-translational modifications, and degradation pathways. Alpha-synuclein misfolds and progressively assembles into oligomers, protofibrils, and fibrils, ultimately forming pathological inclusions and ribbon-like aggregates. Post-translational modifications, including phosphorylation, ubiquitination, and nitration, modulate its structural properties and aggregation propensity, with phosphorylation at serine-129 as a key pathological marker. Alpha-synuclein inclusions co-localize with synphilin-1, Parkin, tau, molecular chaperones, LRRK2, ubiquitin, and cytoskeletal proteins, contributing to neurodegeneration. The ubiquitin-proteasome system mediates clearance of alpha-synuclein aggregates and chaperone-mediated autophagy, with dysfunction in these pathways leading to its accumulation, particularly in the substantia nigra.

3. Mechanisms of Aggregation and Pathology

3.1. Mechanisms of Aggregation

The dual localization of aSyn as a cytosolic and membrane-associated protein highlights its physiological versatility while rendering it susceptible to dysregulation. In this regard, synucleinopathies are defined by abnormalities in the normal cellular distribution of aSyn, leading to its accumulation in aggregated forms[12,113,114]. In such conditions, aSyn undergoes dramatic structural transitions, shifting from alpha-helical-rich states to beta-sheet-rich conformations[115]. This change drives the formation of toxic oligomers, protofibrils, and amyloid fibrils (**Figure 2**)[116,117]. Because toxic aSyn oligomers arise early in aggregation[118], they are considered targets for diagnostic and therapeutic interventions[117]. However, despite extensive research, the molecular mechanisms driving aSyn misfolding remain elusive. Key questions include identifying the factors initiating aggregation, characterizing the toxic structural intermediates, and understanding the cellular conditions facilitating these pathological changes.

The aggregates derived from aSyn exhibit significant heterogeneity across synucleinopathies, with recent studies identifying distinct conformational structures, such as fibrils and ribbons. It has been proposed that these aggregates represent different "strains" analogous to prion strains and exhibit distinct properties, including variations in size, structure, toxicity, lipid-binding efficacy, and

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seeding potential[119–121]. The diversity of aSyn strains may contribute to the clinical heterogeneity observed in synucleinopathies, as each strain potentially drives unique pathological processes.

Environmental factors, including salt concentration, pH, and bacterial endotoxins, have been shown to influence aSyn aggregation and strain formation[122]. *In vivo* mouse model studies reveal that different aSyn strains elicit distinct biological effects[123]. Oligomers and ribbons propagate pathology more efficiently than fibrils, yet only ribbons are associated with phosphorylated aSyn deposits, a hallmark of neurodegeneration. Structural polymorphs of aSyn differ in seeding capacity, toxicity, and propagation dynamics, shaping disease progression and offering potential therapeutic targets[124]. Despite these findings, the strain concept remains unvalidated in human synucleinopathies, and the precise mechanisms driving strain-specific aggregation and toxicity remain elusive.

PTMs add another layer of complexity to regulating aSyn aggregation[125], as described below. Phosphorylation at critical residues is thought to modulate aggregation dynamics, whereas C-terminal truncations—frequently observed in Lewy bodies—are known to enhance aggregation[126]. Additionally, interactions with cellular membranes and lipids play a crucial role, as specific lipid compositions significantly influence aSyn's propensity to aggregate[127]. However, the mechanisms linking these factors to pathological aggregation remain poorly understood. Intriguingly, recent evidence challenges the conventional view that Lewy pathology is central to diseases like DLB, raising questions about the relative contributions of aSyn aggregates to disease progression[128,129].

A major challenge in studying aSyn aggregation is the variability inherent in experimental systems. Recombinant aSyn, commonly used in aggregation studies, often forms distinct strains under laboratory conditions that may lack physiological relevance, complicating comparisons with human-derived aggregates[130]. This variability highlights the necessity for standardized aggregation protocols and careful selection of protein sources to ensure reliable and reproducible findings. Advanced structural techniques, such as cryo-electron microscopy and solid-state nuclear magnetic resonance, are indispensable for elucidating the molecular architecture of aSyn aggregates and bridging the gap between recombinant forms and those extracted from human brain tissue[131,132].

Establishing robust protocols for isolating aggregated aSyn from human brain tissue is a critical research priority[133]. Such protocols are essential for advancing our understanding of pathological aggregates' structural and biochemical properties and comparing these to experimentally generated forms, thereby enhancing the physiological relevance of *in vitro* models. Moreover, they must minimize artefacts for meaningful comparisons with recombinant strains and experimental models[134]. If distinct aSyn strains are identified, they could serve as a foundation for conformation-specific therapeutic strategies. Targeting individual strains might enable more precise interventions, although the coexistence of multiple pathological protein species in the brain poses a significant challenge[135]. In particular, mixed pathologies, where aSyn aggregates coexist with other misfolded proteins like tau or amyloid-beta, will likely require combinatorial approaches to address each pathological species independently [136–140].

Despite these challenges, substantial progress has been achieved in elucidating the mechanisms underlying aSyn aggregation. Future research should focus on refining experimental models to replicate the human brain environment more accurately, integrating insights from diverse systems, and investigating the functional implications of aSyn strain diversity. Such efforts will advance our understanding of synucleinopathies and inform therapeutic development[141]. This multifaceted approach holds promise for uncovering novel therapeutic targets and advancing our understanding of the complex biology underlying synucleinopathies[40].

One key area of investigation is the mechanistic basis of aSyn pathological propagation. Receptor-mediated internalization has gained traction as a plausible model. Several receptors, including neurexin 1b, Aβ precursor-like protein 1, and lymphocyte-activating 3, facilitate the uptake of extracellular aSyn, with the latter showing selective binding to aggregated forms[142,143]. Additionally, prion protein (PrPC) has been implicated in sensing toxic aSyn oligomers, triggering

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synaptic dysfunction[142]. These findings support a "prion-like" propagation mechanism, where aggregated aSyn not only oligomerizes but induces the misfolding of endogenous aSyn, perpetuating its pathological spread across cells.

3.2. Role of Post-Translational Modifications and Metals in α-Synuclein Pathology

PTMs and interactions with metal ions have emerged as critical factors in aSyn pathology due to their profound influence on its structure, function, and aggregation dynamics[144–146]. The amino acid sequence of aSyn contains distinct sites with varying affinities for metal ions, which modulate its folding, aggregation propensity, and interactions with cellular membranes, contributing to its role in synucleinopathies[147]. Among these, copper (Cu(I/II)) binding at the N-terminal region (1MDVFMK6) and histidine 50 (H50) have been shown to accelerate fibril formation *in vitro*, even at micromolar concentrations[148,149]. Similarly, calcium (Ca(I/II)) is implicated in aSyn's role in vesicular transport, with evidence suggesting that Ca(II) and other metals—such as Fe(II), Fe(III), Ni(II), Zn(II), and Mn(II)— preferentially bind to the C-terminal region (119DPNEA125)[150,151]. These metal interactions destabilize monomeric and dimeric aSyn, promoting aggregation and potentially exacerbating disease progression.

PTMs further complicate aSyn pathology by modulating its structural and functional properties[141]. Phosphorylation at serine 129 (S129) is the most extensively studied PTM, though phosphorylation at other sites, such as serine 87 (S87), has also been implicated[152]. Notably, S87 phosphorylation has been shown to reduce fibrillization, suggesting a potential protective role[153,154]. In contrast, phosphorylation of aSyn at Ser129 (pS129) appears to fine-tune neuronal function and activity, reversibly occurring in response to synaptic activity, without direct toxicity[37]. PTMs influence aSyn's metal-binding properties and aggregation propensity, as seen with phosphorylation at tyrosine 125 (Y125) and S129.

N-terminal acetylation, a physiological PTM, enhances Cu(I) binding, increases aSyn's alpha-helical content, and improves its membrane-binding capacity[155]. Recent research has also identified lysine acetylation at residues 6 and 10, which mitigates aSyn aggregation and toxicity[156]. Additionally, PTMs such as ubiquitination, nitration, and sumoylation further regulate aSyn's aggregation dynamics, though their precise roles in synucleinopathies remain poorly understood.

A significant challenge in studying aSyn stems from the reliance on recombinant protein expressed in bacterial systems, which lack eukaryotic-specific PTMs such as N-terminal acetylation[157]. The absence of these modifications raises concerns regarding the physiological relevance of findings derived from non-acetylated aSyn studies, highlighting the need for validation in models that more accurately reflect the native biochemical environment[141].

Critical questions remain regarding PTMs and metal binding in aSyn pathology[158]. For instance, does metal binding to aSyn—particularly Cu and Fe—exacerbate oxidative stress and accelerate neurodegeneration[159]? Moreover, cell-type-specific variations in PTM patterns and their impact on aSyn aggregation and disease propagation remain poorly characterized[128].

Recent findings challenge prevailing models of metal-aSyn interactions, as aSyn purified from blood or brain tissue appears to lack classical metalloprotein properties[160]. This underscores the need for more refined experimental systems and methodologies. Additionally, the potential of PTMs as biomarkers in blood or CSF remains underexplored mainly[161]. Significant challenges in assay sensitivity and specificity hinder progress in this area, underscoring the necessity for advancements in detection methodologies.

Current analytical techniques, including mass spectrometry and antibody-based approaches, are limited in their ability to characterize aSyn PTMs34 comprehensively. Improving these methodologies will be essential to unravel the complex interplay between PTMs and metal interactions[161]. Generating high-quality aSyn variants with specific PTMs will be pivotal for conducting detailed structural and functional analyses.

Future research should prioritize the development of refined model systems that faithfully replicate the cellular environment, allowing for systematic modulation of PTMs and metal

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interactions[162]. Such advancements will be crucial for elucidating the molecular mechanisms underlying aSyn pathology, ultimately providing a foundation for therapeutic innovation. Deep mutational scanning (DMS) is a powerful technique to map sequence-activity relationships through systematic mutagenesis[163]. Recently, DMS studies in yeast revealed that different structural states of aSyn exhibit differential sensitivities to specific mutations, showing that this IDP responds to cellular environment changes[164]. Additionally, oxidative stress has been shown to influence aSyn metabolism, mainly through mitochondrial damage[165]. Furthermore, advances in structural biology techniques, such as cryo-electron microscopy (cryo-EM), in-cell nuclear magnetic resonance (NMR), and super-resolution microscopy, have provided unprecedented insights into aSyn conformations[166–168]. These cutting-edge tools continue to enhance our understanding of aSyn's structure, localization, and function in both physiological and pathological contexts.

3.3. Lewy Bodies and Pathological Inclusions

Although significant synucleinopathies, aSyn aggregates are not in the final stage. LBs and LNs are hallmark pathological features of PD and DLB, characterized as eosinophilic inclusions within neuronal cells. First described in 1912 by Fritz Jakob Heinrich Lewy, these structures comprise dense proteinaceous cores surrounded by radially oriented filaments[169,170]. Subsequent studies revealed that LBs are enriched with ubiquitin [171,172] and aSyn, their principal protein component[7]. Notably, phosphorylated aSyn at serine 129 and other PTMs are consistently detected within LBs, highlighting its critical role in synucleinopathy pathogenesis[173].

The molecular complexity of LBs suggests a multifaceted role in disease pathology. In addition to aSyn, over 90 proteins have been identified within LBs, including synphilin-1[174], Parkin[175], Tau[176], chaperone proteins[63], and leucine-rich repeat kinase 2 (LRRK2)[177], have been identified within LBs. These proteins encompass structural and binding elements, components of the ubiquitin-proteasome system, and factors involved in cellular stress responses, cytoskeletal integrity, cell cycle regulation, and intracellular signalling pathways[178]. Advanced imaging techniques have revealed the presence of membranous components, including mitochondria and lysosomes, within Lewy LBs. Interestingly, these studies suggest a relatively low abundance of aSyn fibrils, indicating a more complex assembly process than previously understood[129]. This biochemical heterogeneity highlights the need for further investigation into the mechanisms governing LB formation and their biological significance in synucleinopathies.

The anatomical distribution of LBs aligns with the clinical symptoms observed in PD and DLB[179]. LBs are predominantly located in dopaminergic neurons within the substantia nigra (SN), contributing to the motor deficits characteristic of PD[180,181]. Additionally, they are present in glutamatergic pyramidal neurons of the limbic system and neocortex and cholinergic neurons in the basal forebrain, correlating with cognitive and behavioural symptoms[182–184]. This selective vulnerability highlights the complex interplay between LB pathology and neuronal populations.

The functional role of LBs remains an area of ongoing debate. Studies diverge on whether LBs exert toxic, inert, or protective effects. Some evidence suggests a correlation between LB burden and symptom severity[185–187], whereas other findings indicate that neurons harbouring LBs may exhibit resilience against degeneration[188]. Several hypotheses have been proposed to reconcile these discrepancies, including (a) the rapid death of neurons prone to LB formation, leaving behind LB-resistant neurons; (b) subpopulations of LBs with varying toxicities; and (c) the possibility that LB formation is a neuroprotective response to toxic intermediates. Notably, familial PD cases that lack LB pathology provide support for the latter hypothesis[189].

A combination of biochemical and functional factors likely contributes to LB formation[190]. Neurons with high metabolic demands, calcium dysregulation, and reliance on protein degradation pathways may be predisposed to LB accumulation[191]. Furthermore, interactions with other neuropathological hallmarks, such as Tau aggregation, add further complexity to the picture[192]. Although peripheral aSyn aggregation has been observed, the clinical significance of these findings remains unclear.

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Advancing our understanding of LBs necessitates high-resolution studies of their biochemical composition and molecular architecture[193]. Techniques such as mass spectrometry and selective antibodies are instrumental in identifying key components and their interactions[194]. Elucidating the modulators of LB formation could reveal new therapeutic targets[190]. Access to high-quality post-mortem brain tissues from well-maintained brain banks remains essential for these investigations.

Developing aSyn-specific ligands for PET imaging offers a promising tool for tracking LB pathology *in vivo[195,196]*. Such imaging modalities could provide crucial insights into the spatial and temporal dynamics of LB progression. In parallel, patient-derived iPS cells and three-dimensional cell culture models serve as valuable platforms for recapitulating LB formation and evaluating therapeutic interventions[197]. However, further validation is required to enhance the reliability of these models.

By integrating advanced biochemical, imaging, and model-based approaches, researchers can unravel the complexities of LBs, paving the way for targeted therapies and improved diagnostic tools.

3.4. Genetic and Phenotypic Diversity in Synucleinopathies

The three primary synucleinopathies—PD, DLB, and MSA—are characterized by overlapping yet distinct clinical and pathological features[198]; despite well-documented clinical distinctions, the mechanisms driving the phenotypic diversity among these diseases remain poorly understood[199]. Genetic and environmental factors and ageing play significant roles in modulating their pathogenesis, but the interplay between these elements remains a key area of investigation[200].

Although most synucleinopathy cases are sporadic, genetic studies have illuminated their molecular underpinnings[34,201]. Early research identified mutations in the *SNCA* gene, which encodes aSyn, as the first genetic link to familial PD[167,202]. Mutations, such as A53T, E46K, and A30P, along with rare *SNCA* locus multiplications, directly increase aSyn levels or alter its aggregation properties, thereby promoting pathology[202]. However, it remains unclear why these mutations predispose individuals to specific synucleinopathies, such as PD versus DLB or MSA.

Genome-wide association studies (GWAS) have expanded our understanding of genetic contributions by identifying variants associated with PD[203], DLB[204], and MSA[205]. These findings underscore the complexity of genetic risk, as these diseases involve multiple low-penetrance variants rather than single high-penetrance mutations. For example, Leucine-rich repeats kinase 2 (*LRRK2)* and *Parkin RBR E3 ubiquitin-protein ligase* (*PARK2*) mutations contribute to the PD spectrum but exhibit phenotypic variability[34,201]. *LRRK2* variants, the most common genetic cause of familial PD, are not always accompanied by Lewy pathology, as evidenced by post-mortem analyses showing pure nigral degeneration in some carriers[206]. Similarly, *PARK2* mutations, commonly linked to early-onset parkinsonism, are often observed in cases lacking Lewy body pathology[6]. These observations highlight the heterogeneity of synucleinopathies and emphasize the need for deeper investigations into genotype-phenotype correlations to understand disease mechanisms better.

Another key genetic link involves mutations in the *Glucosylceramidase Beta 1 (GBA1)* gene, which encodes glucocerebrosidase, an enzyme critical for lysosomal function[207]. Heterozygous *GBA1* mutations significantly increase the risk of developing PD and DLB and may contribute to MSA[208]. The association between glucocerebrosidase dysfunction and aSyn pathology suggests that lysosomal impairments and autophagy defects play pivotal roles in synucleinopathies[209,210]. This connection underscores the broader role of lysosomal biology in regulating aSyn homeostasis and pathology.

Genetic studies of synucleinopathies reveal both shared and disease-specific mechanisms[211]. For instance, the role of aSyn aggregation in PD, DLB, and MSA suggests overlapping pathogenic pathways, while disease-specific genetic factors may dictate phenotypic expression[212]. Interestingly, duplication or triplication of the *SNCA* gene is associated with early-onset parkinsonism and atypical symptoms, including hallucinations, cognitive impairment, and dementia[213,214]. However, the rarity of *SNCA* mutations highlights the influence of additional

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factors, such as epigenetic modifications, environmental exposures, and cellular context, in modulating disease phenotypes[215].

Epigenetic mechanisms, such as DNA methylation and histone modifications, are emerging as critical regulators of synucleinopathies[216,217]. Epigenetic modifications may regulate aSyn expression, modulate neuronal vulnerability, and shape immune responses, shedding light on the heterogeneity of these diseases[216]. The plasticity of the epigenome presents a promising opportunity to identify modifiable risk factors and guide the development of personalized therapeutic strategies.

Integrating genetic findings with neuropathological and mechanistic studies is essential to advancing our understanding of synucleinopathies[167]. Patient-derived materials like iPS cells allow researchers to study disease-relevant genetic variants in the appropriate cellular contexts[218]. Additionally, improved *in vivo* models incorporating multiple genetic and environmental factors are needed to recapitulate the complexity of human synucleinopathies[123]. Furthermore, defining subtypes of synucleinopathies through genetic and epigenetic insights offers significant potential for advancing personalized medicine[219]. For example, patients harbouring *GBA1* mutations may benefit from therapies targeting lysosomal dysfunction, whereas those with *LRRK2* variants could respond favourably to kinase inhibitors[220]. This stratified approach can potentially enhance therapeutic efficacy while reducing the variability in treatment outcomes.

The emerging field of multi-omics, which integrates genomics, transcriptomics, proteomics, and metabolomics, holds promise for uncovering previously unrecognized pathways underlying synucleinopathies[221]. Coupling these approaches with advanced computational tools enables researchers to identify novel biomarkers and therapeutic targets, providing new insights and addressing critical knowledge gaps in the field.

4. Propagation and Spreading

4.1. The Role of α-Synuclein Spreading in Pathology

The biological relevance of aSyn spreading has garnered increasing support, with evidence suggesting that it is more than a secondary phenomenon. Notably, Lewy pathology has been detected in grafted neurons of PD patients, indicating intra-brain transmission of aSyn pathology[118,222]. Animal models further corroborate the ability of misfolded aSyn to propagate across neural circuits. However, confirmation of aSyn transmission at endogenous levels in human synucleinopathies remains elusive[223].

The propagation of aSyn pathology across interconnected brain regions is hypothesized to occur by converting physiological aSyn into aggregated or pathological forms (**Figure 3**)[224,225]. This spreading is proposed to occur in a 'prion-like' manner, where misfolded aSyn serves as a template for the conformational transformation of native aSyn. However, this concept remains debated, with unresolved questions about its physiological relevance and pathological mechanisms. Several pathways for aSyn transmission have been proposed, including direct diffusion across cell membranes, secretion via extracellular vesicles, and intercellular transfer through tunnelling nanotubes[226]. Additionally, aSyn fibrils can interact with heparan sulfate proteoglycan chains on the plasma membrane, facilitating macropinocytosis as a mechanism for cellular uptake[227]. These processes exhibit cell-type specificity, predominantly in neurons and oligodendrocytes[66,228].

The extent to which aSyn pathology disseminates among different cell types, including neurons and glia, is not yet fully understood[40,229]. Microglia have been implicated in the uptake and processing of aSyn fibrils through phagocytosis, followed by lysosomal cleavage mediated by asparagine endopeptidase (AEP), potentially contributing to the propagation of pathology[230]. Furthermore, whether aSyn spreads from peripheral tissues, such as the gut or olfactory bulb, to the brain—or vice versa—remains unproven in humans[53,54,231]. Current insights into these processes primarily derive from animal models, which may not fully replicate human synucleinopathies.

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A critical question in the field is whether aSyn spreading represents a physiological process or a pathological epiphenomenon[223]. The involvement of PTMs in modulating aSyn's aggregation and transmissibility is an area of active investigation[232]. For example, phosphorylation at S129 and truncations in the C-terminal region may enhance aSyn's seeding potential, yet their precise roles remain unclear[233]. Moreover, whether synucleinopathies should be classified as 'prion' disorders or primarily as protein aggregation diseases continues to be debated[234,235].

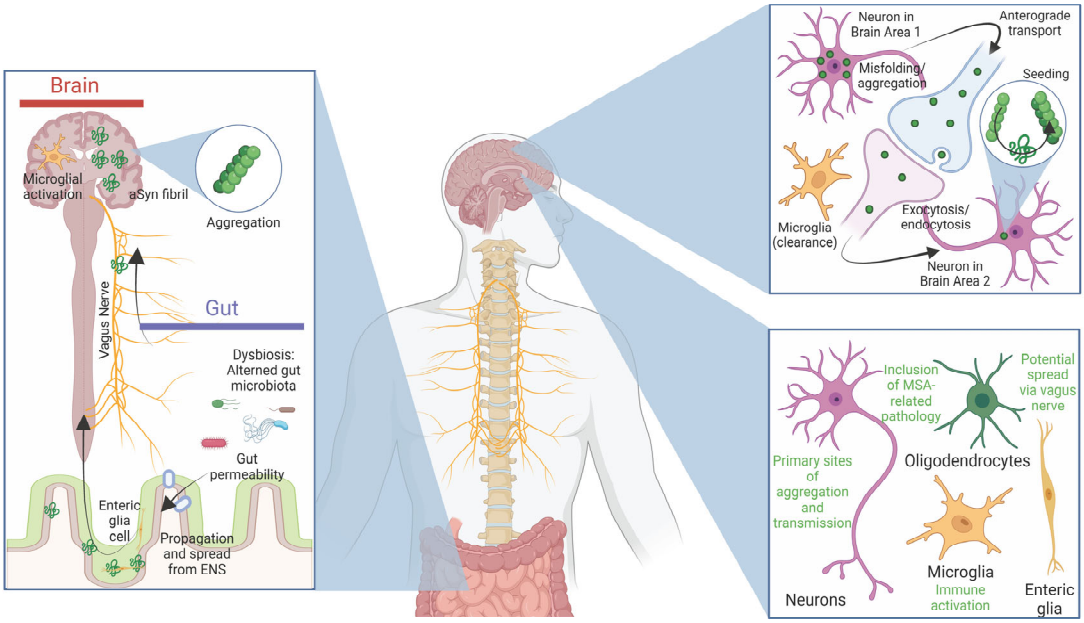

Figure 3. | Propagation and cellular interactions of alpha-synuclein pathology. Alpha-synuclein aggregates propagate between neurons through anterograde transport, exocytosis, endocytosis, and seeding mechanisms, contributing to disease spread across brain regions. Microglia play a crucial role in alpha-synuclein clearance, but their activation may also exacerbate neuroinflammation. In multiple system atrophy (MSA), oligodendrocytes harbour alpha-synuclein inclusions, further driving disease pathology. The gut-brain axis is implicated in early alpha-synuclein aggregation, with enteric glial cells, increased gut permeability, and dysbiosis influencing neurodegenerative processes. Transmission via the vagus nerve has been proposed as a route for gut-derived alpha-synuclein pathology to reach the central nervous system, highlighting a potential disease initiation and progression mechanism.

Addressing these knowledge gaps requires advanced cellular and animal models that accurately reflect endogenous aSyn expression levels and the cellular complexity of the human brain[141].[236,237]. Such models should mimic endogenous aSyn expression levels and the cellular diversity of human brains. Investigating how aSyn is released, internalized, and trafficked between cells and how PTMs influence these processes could identify critical intervention points[111]. High-resolution imaging and proteomic approaches will be crucial for visualizing the dynamics of aSyn spreading and mapping its molecular interactions.

Therapeutic strategies targeting aSyn spreading must carefully balance its potential dual role in physiology and pathology. While blocking intercellular transmission may mitigate disease progression, it could also interfere with normal aSyn function[40]. Thus, selectively targeting pathological aSyn conformations while preserving its normal function is crucial. Advancements in experimental models and innovative technologies will be instrumental in refining our understanding of aSyn transmission and developing effective therapeutic interventions for synucleinopathies.

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4.2. Immune Responses and the Microbiome in Parkinson's Disease Pathogenesis

Post-mortem studies of PD brains consistently reveal pronounced microgliosis in regions affected by aSyn pathology, including the SN, hippocampus, and cortex[238–241]. These findings suggest that microglial activation is not merely a consequence of neuronal loss but represents an active and potentially maladaptive response to aSyn pathology. Neurons release aSyn, especially in aggregated forms, which elicit immune responses by engaging microglia and macrophages to clear extracellular aSyn[96,242]. However, the efficiency of these immune cells depends on the aggregation state of aSyn, with misfolded species inducing stronger and potentially detrimental inflammatory responses[243], in part through activation of the NLRP3 inflammasome in microglia[244,245].

Microglia play a dual role in PD pathogenesis. On the one hand, they may protect neurons by clearing toxic aggregates of aSyn; on the other, chronic microglial activation exacerbates neuroinflammation, amplifying pro-inflammatory signals and promoting neurodegeneration[96,242,246]. This delicate balance between neuroprotection and neurotoxicity underscores the complexity of microglial responses in PD. Persistent inflammation may establish a self-perpetuating cycle, wherein the immune response to aSyn pathology accelerates neuronal damage, leading to further aSyn release and sustained microglial activation[247]. This pro-inflammatory milieu, driven by pathological aSyn aggregates, can facilitate peripheral immune cell infiltration through chemotaxis or BBB disruption, further exacerbating neuroinflammation[248].

Emerging evidence suggests that the gut microbiome is critical to PD pathogenesis. Studies in PD patients consistently report alterations in gut microbial composition, including reduced bacterial diversity and signs of gastrointestinal inflammation[249–251]. PD-associated microbiota is characterized by increased *Akkermansia* and *Bifidobacterium* and decreased *Roseburia* and *Faecalibacterium*. Besides, the depletion of butyrate-producing bacteria and excessive mucus degradation by *Akkermansia* may contribute to intestinal inflammation and increased gut permeability, facilitating the translocation of harmful metabolites into the enteric nervous system (ENS)[252]. Dysbiosis-induced inflammation may drive aSyn accumulation in the gut, promoting its propagation to the brain via the gut-brain axis and triggering neuroinflammation, dopaminergic neuron loss, and ultimately PD progression[253]. Moreover, gut microbiota alterations may serve as biomarkers and disease modifiers, as microbiome imbalances can induce aSyn aggregation in animal models, highlighting a possible causal link [254] and the implementation of microbiome-based therapies[255].

The hypothesis that aSyn pathology originates in peripheral tissues, such as the gut or nasal epithelia, before spreading to the CNS has gained increasing support[256–258]. Phosphorylated aSyn aggregates have been identified in the ENS of PD patients[259,260], and experimental models have demonstrated that exogenous aSyn fibrils can propagate from the gut to the brain via the vagus nerve[261,262]. Afferent fibres of the vagus nerve are capable of sensing microbiota-derived metabolites, such as lipopolysaccharide (LPS) and short-chain fatty acids (SCFAs), and transmitting these signals to the brainstem via the nucleus tractus solitarii, a component of the dorsal vagal complex[263]. This pathway may contribute to increased susceptibility to PD[264].

Epidemiological studies further suggest that surgical interventions, such as appendectomy and vagotomy, are associated with a reduced risk of PD[265–268]. Despite these advances, significant gaps remain in understanding the interplay between the gut, the immune system, and aSyn pathology[111]. Key outstanding questions include whether peripheral immune cells encounter aSyn aggregates before microglial activation in the CNS, the mechanisms underlying peripheral-to-central communication during disease progression, and the role of post-translational modifications in modulating immune responses.

Additionally, the direct interaction between microglia and neurons warrants further investigation. The discovery of Lewy body-like structures, including phosphorylated S129-positive inclusions, within microglial cells suggests a direct pathological link between neurons and microglia[233]. However, the consequences of these inclusions for microglial function remain unknown. Do microglial cells with aSyn inclusions remain viable and functional, or do they succumb

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to cell death? Additionally, why do aSyn inclusions exhibit distinct characteristics across cell types, such as microglia and oligodendrocytes in multiple system atrophy (MSA)?

Addressing these questions requires advanced models and methodologies. Patient-derived organoids, *in vivo* imaging, and molecular profiling technologies will be essential for characterizing immune responses in experimental and clinical contexts[269]. These approaches could reveal the dynamic interactions between neuroinflammation and the gut-brain axis in PD pathogenesis, providing key insights for diagnostic and therapeutic development. Identifying precise modulators of inflammation and aSyn pathology, whether through microbial interventions, anti-inflammatory strategies, or targeted therapies, represents a promising avenue for mitigating disease progression in synucleinopathies[270].

5. Models of Synucleinopathies

5.1. Animal Models of Synucleinopathies Through α-Synuclein Expression

The development of animal models for studying synucleinopathies has been pivotal in advancing our understanding of aSyn aggregation, neuronal dysfunction, and neurodegeneration[34,151]. These models, primarily based on the expression of wild-type or mutant aSyn, successfully replicate key features of PD and related disorders. However, while early expectations envisioned comprehensive models fully mirroring human synucleinopathies, most only recapitulate specific facets of αSyn biology[271]. Despite these limitations, they remain invaluable tools for dissecting molecular pathways and evaluating therapeutic interventions.

A wide range of transgenic models has been created, including worms (*C. elegans*), flies (*D. melanogaster*), mice, and rats, each offering unique advantages[272]. Invertebrate models facilitate high-throughput genetic and pharmacological screening, bridging the gap between cell-based and whole-organism studies[273–276]. Mammalian models, particularly transgenic mice, are commonly employed to investigate tissue-specific aSyn expression using neuron-specific promoters[277]. While these models have significantly enhanced our understanding of αSyn aggregation and neurodegeneration, they often fail to replicate human disease pathology fully.

Viral vector-mediated models utilizing adeno-associated viruses (AAVs) or lentiviruses offer precise spatiotemporal control of aSyn overexpression via stereotactic brain injections[278,279]. These models facilitate targeted investigations of specific brain regions and neural circuits, enhancing their translational relevance. Additionally, pre-formed fibrils (PFFs) of aSyn represent a significant advancement, as their injection into the brain induces the aggregation of endogenous aSyn, providing a robust platform for studying the propagation and pathogenic mechanisms of aSyn pathology[280,281]. PFF models effectively mimic the prion-like spreading of αSyn pathology, a hallmark of disease progression in PD and related synucleinopathies[282,283], thus allowing interventional studies, such as neuromodulation with optogenetics [284] or engineered binding proteins intended to neutralize aSyn[285]. Notably, PFF inoculation in the duodenal wall of mice led to gastrointestinal deficits and enteric nervous system alterations, reinforcing the role of peripheral synucleinopathy in early PD. Furthermore, aged mice, but not younger ones, exhibited midbrain pathology and motor deficits following αSyn fibril inoculation, highlighting age-dependent susceptibility to neurodegeneration[286].

Despite significant technological advancements, existing models face notable limitations. While many transgenic models successfully induce aSyn aggregation, the resulting inclusions often fail to replicate the ultrastructural and biochemical characteristics of human LBs[287]. The reasons for this discrepancy remain unclear and warrant further investigation. Similarly, consistent neurodegeneration, particularly of dopaminergic neurons in the substantia nigra, is rarely observed, leaving critical questions about selective neuronal vulnerability unresolved[288]. Addressing these gaps is essential for developing more accurate and predictive models that better reflect the complexity of human synucleinopathies.

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Several neurotoxin-induced models replicate specific features of PD, but they often fail to capture the slow, progressive, and irreversible neurodegeneration and do not induce αSyn pathology.[289]. Recently, a murine model using intranigral administration of the neurotoxin BSSG was developed, successfully mimicking many hallmark features of PD, including motor and non-motor symptoms, nigrostriatal neurodegeneration, neuroinflammation, oxidative stress, and pathological aSyn aggregation and propagation. This model presents a promising platform for studying disease mechanisms and testing therapeutic interventions[290,291].

Emerging technologies like CRISPR/Cas9 genome editing promise to create refined PD and synucleinopathies models. These tools allow precise genetic modifications to introduce disease-relevant mutations, replicate human-like expression profiles, and manipulate regulatory elements[292]. Incorporating PTMs and protein interactors into these models could enhance their physiological relevance. Insights from tauopathy models emphasize the need to minimize artefacts and rigorously characterize models to ensure reproducibility and reliability[293], setting a benchmark for advancing aSyn research. Knock-in mouse models are valuable for studying neuroinflammation, neurodegeneration, and αSyn-mediated gastrointestinal and olfactory dysfunctions [294][295].

Expanding model development to non-human primates presents a promising avenue for synucleinopathy research. Knock-in primate models can better represent human brain circuits and physiological mechanisms, enhancing translational potential[296]. These models could serve as more accurate platforms for testing therapeutic strategies, helping to bridge the translational gap between preclinical findings and clinical applications.

Future research should focus on developing models that comprehensively integrate key pathological features, including aSyn aggregation, Lewy body formation, neurodegeneration, and cell-type-specific vulnerabilities[272]. Leveraging advanced genetic tools alongside high-resolution imaging, proteomics, and electrophysiology will provide deeper insights into the multifaceted roles of aSyn in health and disease. These integrated approaches are essential for identifying novel therapeutic targets and accelerating the development of disease-modifying treatments for synucleinopathies.

5.2. Cellular Models of α-Synuclein Toxicity and Aggregation

Cellular models have been pivotal in elucidating the biology, aggregation dynamics, and toxicity of aSyn. These models span a broad spectrum, including yeast, neuronal and non-neuronal mammalian cell lines, primary neuronal cultures, and patient-derived iPS cells, offering a simplified yet versatile approach to studying aSyn pathology[287,297]. Transient or stable expression systems facilitate the investigation of wild-type and PD-associated mutant forms of aSyn, enabling cost-effective and ethically viable research avenues.[298,299]. However, their ability to fully recapitulate the complexity of human synucleinopathies remains limited.

To induce aSyn aggregation, cellular models often rely on exogenous stressors such as 1-methyl-4-phenylpyridinium, rotenone, paraquat, or proteasome inhibitors[300], as overexpression alone frequently fails to form inclusions[301,302]. A notable advancement involves adding extracellular PFFs to primary neuronal cultures, triggering the recruitment of endogenous aSyn into ubiquitinated and hyperphosphorylated aggregates resembling pathological hallmarks of synucleinopathies[303]. These models have significantly contributed to uncovering links between aSyn and mitochondrial dysfunction, oxidative stress, and impaired protein degradation pathways[304–306]. Furthermore, cellular systems have facilitated the identification of surface proteins that interact with aSyn [142,143] and supported studies on glial degeneration in MSA, mainly through the co-expression of aSyn and p25α in oligodendroglial cell lines[307].

Despite these contributions, several limitations persist. The precise mechanisms underlying aSyn aggregation and cytotoxicity remain incompletely understood. Soluble aSyn oligomers are hypothesized to mediate cellular dysfunction and death during the early stages of aggregation, yet their exact roles remain elusive[306,308,309]. In MSA, accumulating aSyn in oligodendrocytes

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presents unresolved questions regarding its origin and pathogenic mechanisms[310]. Moreover, proteins such as synphilin-1 [55,311] and p25α [58,312] have been implicated in modulating aSyn aggregation. However, their precise roles remain unclear, pointing to the likely existence of additional yet unidentified regulatory factors that influence the aggregation process.

Methodological constraints further challenge the field. The widespread use of antibodies and fluorescent tags for detecting aSyn aggregation may inadvertently alter its biological properties, potentially introducing experimental biases. Addressing these issues requires the development of more specific antibodies and non-invasive tagging strategies to enable precise characterization of aSyn species in physiologically relevant contexts. Moreover, current cell models produce LB-like inclusions rather than proper LB, emphasizing the need for refined approaches to better mimic disease pathology[190,306,313]. Although iPS cell-derived models hold promise, epigenetic alterations introduced during reprogramming may compromise their fidelity in replicating disease phenotypes.

Future efforts should focus on developing models that integrate genetic, epigenetic, and environmental factors contributing to synucleinopathies. Advanced technologies, such as CRISPR/Cas9 for precise genetic editing and organoid-based 3D cultures, can bridge the gap between traditional cellular models and in vivo systems[314]. Refining existing tools and incorporating novel approaches will enable the creation of more physiologically relevant models, enhancing our understanding of aSyn pathology and accelerating the discovery of targeted therapeutic strategies.

6. Towards Diagnostic and Therapeutic Strategies

6.1. Biomarkers for Diagnosis

The diagnosis of PD primarily relies on the clinical evaluation of motor symptoms, which typically manifest at advanced disease stages[315]. These motor features often overlap with those of other synucleinopathies, further complicating early diagnosis. Although genetic testing offers valuable insights into familial cases, most PD cases are sporadic, limiting its widespread applicability[316]. These challenges highlight the urgent need for reliable biomarkers to enable early detection, differentiate PD from related disorders, and effectively track disease progression.

aSyn has emerged as a promising biomarker candidate (**Figure 4**). Detectable in CSF, plasma, blood, saliva, and even tear fluid, aSyn and its PTM forms have been extensively studied for their diagnostic potential[317–319]. Among these, CSF-based assays have shown the greatest promise, with studies suggesting that CSF's ratio of aSyn isoforms could serve as a sensitive and specific diagnostic tool[320–322]. PTMs of aSyn, including phosphorylation and truncation, have also been implicated as potential biomarkers measurable in CSF and blood[99,323].

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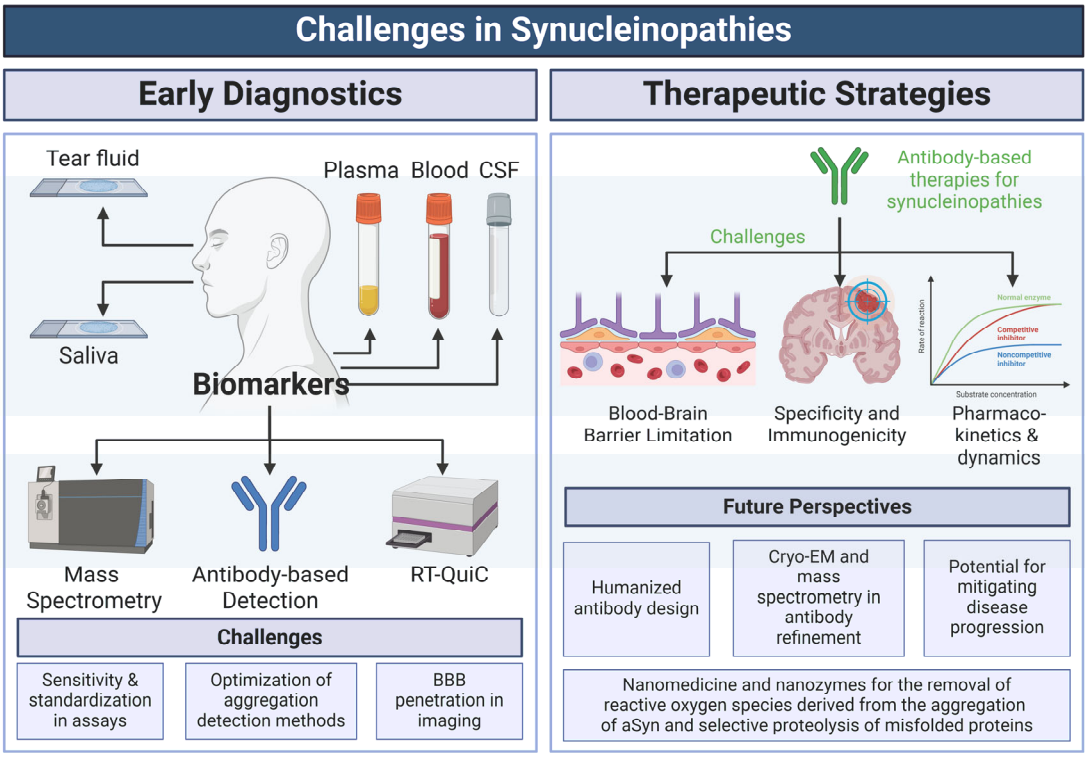

Figure 4. Challenges and future perspectives in synucleinopathy diagnostics and therapeutics. Early diagnosis of synucleinopathies relies on the detection of alpha-synuclein biomarkers in cerebrospinal fluid, saliva, plasma, and tear fluid using techniques such as mass spectrometry, antibody-based detection, and real-time quaking-induced conversion (RT-QuIC). However, challenges remain in assay sensitivity, standardization, and optimizing aggregation detection methods. Imaging approaches are further limited by blood-brain barrier penetration. Therapeutic strategies, including antibody-based therapies, face hurdles related to specificity, immunogenicity, pharmacokinetics, and pharmacodynamics. Future perspectives involve the development of humanized antibodies, advanced cryo-electron microscopy (cryo-EM) and mass spectrometry techniques for antibody refinement, and the use of nanomedicine and nanozymes to target reactive oxygen species and selectively degrade misfolded alpha-synuclein aggregates, offering potential avenues for disease modification.

Despite significant advancements, several challenges remain. A critical hurdle is the development of sensitive and standardized assays to quantify aSyn and PTMs across diverse biological fluids accurately. Cutting-edge methodologies, such as advanced mass spectrometry and optimized antibody-based detection techniques, ensure reproducibility and inter-laboratory consistency[324]. These assays must account for variations in aSyn levels across sample types, disease stages, and patient populations to enhance their diagnostic and prognostic utility.

The second major challenge lies in advancing PET imaging for aSyn. Developing PET tracers capable of detecting various forms of aSyn would enable non-invasive, longitudinal studies in patients and at-risk individuals. However, selectivity and BBB penetration remain significant obstacles to practical imaging[325,326]. Overcoming these limitations could revolutionize the *in vivo* tracking of aSyn pathology, providing insights into disease progression and therapeutic responses.

A third area of focus involves leveraging innovative aggregation detection techniques. Methods such as real-time RT-QuiC and protein misfolding cyclic amplification have shown high sensitivity in detecting aggregated, pathology-associated aSyn. These approaches rely on the amyloid-specific interactions of aSyn fibrils with thioflavin T, producing characteristic emission spectra. While RT-QuiC has demonstrated promising results in detecting PD using CSF samples, further optimization is required to enhance its reliability for widespread clinical use[327,328].

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Integrating these tools into clinical and research settings is essential for advancing PD diagnostics. Establishing standardized protocols for aSyn measurement across various fluids and disease stages will improve the accuracy of disease burden and progression assessments. Moreover, developing these biomarkers offers the potential for early intervention, improved differentiation of PD from related disorders, and precise therapeutic efficacy monitoring.

Addressing these challenges will require a multidisciplinary effort to refine detection technologies, optimize imaging methodologies, and enhance experimental reproducibility. Longitudinal studies employing these refined biomarkers can potentially deepen our understanding of PD pathophysiology, ultimately paving the way for more targeted and effective therapeutic strategies that improve patient outcomes.

6.2. Therapeutic Strategies

Antibodies are essential basic and clinical research tools widely employed in immunostaining, immunoblotting, immunoprecipitation, and diagnostics. Recently, their therapeutic potential has been explored for neurodegenerative diseases, including PD and related synucleinopathies[329,330]. With their high specificity, strong antigen-binding properties, and extended half-life, antibodies are promising candidates for immunotherapy. Notably, conformation-specific antibodies targeting aggregated forms of aSyn have shown efficacy in cell and animal models, with several progressing through clinical trials[331–334]. However, despite their potential, antibody-based therapies face significant challenges that must be overcome to realize their full therapeutic potential. One major limitation is their restricted ability to cross the BBB, as their large molecular size hinders access to intracellular and CNS targets. Additionally, antibodies with low homology to human proteins may provoke immune responses, leading to neutralization and reduced efficacy. To address this, ongoing efforts focus on developing humanized antibodies to minimize immunogenicity and enhance therapeutic safety.

Another critical challenge is identifying the optimal aSyn conformation to target. While aggregated forms such as oligomers and fibrils are pathological hallmarks of synucleinopathies, their precise roles in disease progression remain controversial. Conformation-specific antibodies have shown promise; however, a deeper understanding of the molecular mechanisms driving aSyn pathology is essential to prioritize therapeutic targets. For instance, while most therapeutic antibodies in clinical trials target the C-terminal region of aSyn, truncation of this region has been implicated in promoting aggregation[335]. Furthermore, PTMs that modulate aSyn structure and aggregation propensity must be carefully considered to enhance antibody specificity and therapeutic efficacy.

Pharmacokinetics and pharmacodynamics pose additional challenges in developing antibody-based therapies for synucleinopathies. Key factors must be optimised to maximise therapeutic efficacy, including antibody binding affinity, avidity, cellular internalization, and stability. One promising approach involves developing smaller antibody fragments, such as single-chain variable fragments (scFvs)[336], which exhibit improved BBB permeability and intracellular delivery, thereby increasing therapeutic potential[329,337]. Additionally, therapeutic antibodies must selectively neutralize pathological aSyn species while preserving the physiological functions of aSyn to minimize off-target effects and ensure safety.

Robust preclinical and clinical evaluations are essential for refining antibody design and establishing therapeutic efficacy against synucleinopathies. Longitudinal studies are particularly critical for prioritising oligomeric or fibrillar aSyn forms to maximize clinical impact. Advanced technologies such as cryo-EM, mass spectrometry, and cutting-edge cellular models will play a pivotal role in elucidating the structural basis of antibody-antigen interactions and guiding the optimization of antibody engineering. Overcoming these challenges through innovative design and rigorous validation will be critical to unlocking the full therapeutic potential of antibody-based strategies. Such advancements promise transformative treatments capable of mitigating disease progression and significantly improving patient outcomes.

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Another approach includes developing anti-aggregation therapies (**Figure 4**). In this regard, a deeper understanding of aSyn structure and its cellular uptake is crucial for developing selective molecular inhibitors to mitigate aggregation [338] and prion-like propagation[339]. Recently, CBP/p300 inhibitors have been proposed as potential disruptors of pathological aSyn accumulation in dopaminergic neurons[340], while the BBB-penetrating inhibitor AZD3759 has demonstrated the ability to attenuate prion-like aSyn propagation in mice[341]. Moreover, small aromatic compounds such as SynuClean-D have been shown to inhibit aggregation across multiple aSyn strains[342,343]. Combining *in silico* and *in vitro* models has provided modern strategies to design small molecules targeting secondary nucleation, further advancing therapeutic development[344]. Furthermore, recent studies suggest that promoting aSyn clearance through physiological reactivation of the perivascular fluid transport network, also known as the glymphatic system, may offer a novel therapeutic approach. The glymphatic system plays a crucial role in clearing neurotoxins, including aSyn, and its modulation could represent a viable strategy for reducing the aSyn burden in neurodegenerative diseases[345–348].

Nevertheless, another strategy involves the use of new technologies such as nanomedicine (the application of nanotechnology in medicine), which has achieved significant results in the development of selective enzyme-type catalytic nanostructures with properties for the removal of reactive oxygen species derived from the aggregation of aSyn, as well as with the capacity for the selective proteolysis of misfolded proteins[349]. Although some of these proposals are in the early stages, the deepening of our knowledge concerning the pathological processes of synucleinopathies and advances in nanomedicine could allow the development of potential treatments for these currently incurable diseases.

7. Discussion

The complex and multifaceted biology of aSyn has placed it at the centre of synucleinopathy research. However, significant challenges persist in fully elucidating its role in disease pathogenesis and progression. This review synthesizes insights into aSyn's structure, aggregation dynamics, and cellular interactions, highlighting critical knowledge gaps and outlining future research and therapeutic directions. Addressing these challenges, the field can move closer to unravelling the complexities of aSyn biology and developing effective treatments for synucleinopathies.

A fundamental challenge in synucleinopathy research is deciphering the diverse aggregation states of aSyn and their effects on cellular function. aSyn aggregates, including oligomers, fibrils, and ribbons, adopt distinct conformations and exert varied biological effects, potentially functioning as "strains" contributing to disease heterogeneity[119,120,350,351]. Advanced methodologies such as cryo-electron microscopy (cryo-EM) and single-molecule imaging have significantly improved structural characterization in both experimental models and human-derived samples. However, establishing clear links between specific structural forms, clinical phenotypes, and disease progression remains an urgent objective. Further refinement of these tools and their application in longitudinal studies could provide deeper insights into the mechanisms driving aSyn aggregation and its pathological diversity, facilitating the development of targeted therapeutics.

The second challenge centres on understanding the relationship between aSyn and cellular protein homeostasis mechanisms. Dysfunctions in chaperone-mediated autophagy, macroautophagy, and the ubiquitin-proteasome system contribute to aSyn accumulation and aggregation[87,89]. Recent studies have shown that activating the ubiquitin-proteasome system could dismantle disease-related proteins such as aSyn[352]. Emerging technologies, such as CRISPR-based gene-editing tools and high-throughput proteomics, offer promising avenues to identify key molecular players involved in aSyn degradation[353–355]. Therapeutically targeting these pathways holds significant potential for restoring protein homeostasis and mitigating the neurodegenerative processes underlying synucleinopathies.

The third challenge lies in developing reliable biomarkers for early diagnosis and disease monitoring. The detection of aSyn in CSF, blood, and other biological fluids has shown considerable

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potential, particularly in identifying post-translationally modified species linked to pathology[320,324]. However, the lack of standardized and validated detection assays across laboratories remains a significant barrier to reproducibility and clinical translation. Advanced techniques, such as RT-QuiC and PMCA, have demonstrated exceptional sensitivity in detecting aggregated aSyn species[327,328]. Expanding these methodologies to longitudinal studies and at-risk populations could revolutionize early diagnosis and enhance disease monitoring, paving the way for more effective interventions.

The fourth challenge involves advancing *in vivo* imaging of aSyn pathology using PET tracers[356,357]. These tools present a transformative opportunity to visualize aSyn pathology in real-time, offering critical insights into disease progression's spatial and temporal dynamics. However, challenges such as BBB penetration and tracer specificity hinder their effectiveness[195,326]. Overcoming these limitations requires advances in molecular imaging techniques and the development of high-affinity, selective radioligands. Enhancing PET imaging capabilities will improve diagnostic accuracy and enable more precise monitoring of therapeutic responses in clinical settings.

The fifth challenge involves optimizing immunotherapies targeting aSyn[358,359]. Conformation-specific antibodies have demonstrated promise in preclinical and clinical studies. However, their efficacy remains limited by challenges such as BBB penetration and specificity for pathological forms of aSyn[329,334,360]. Strategies to humanize antibodies, improve delivery mechanisms, and increase specificity for pathological forms are crucial. Complementary therapeutic approaches, including small-molecule inhibitors, peptides, and RNA-based interventions[361], can modulate aSyn aggregation and toxicity. Determining which aSyn species—oligomers, fibrils, or other forms—should be targeted remains a key question in optimizing immunotherapeutic strategies.

The sixth challenge explores the complex interplay between peripheral immune responses, the gut microbiome, and central aSyn pathology. aSyn is a small, highly dynamic protein composedAlterations in the gut microbiota have promoted aSyn aggregation and neuroinflammation, underscoring the gut-brain axis as a key modulator of disease progression[249,362,363]. Innovative therapeutic strategies, such as probiotics, prebiotics, faecal microbiota transplantation, dietary interventions, and gut-specific pharmacological agents, are being explored for their potential to influence these processes. Further research into systemic interactions between peripheral and central mechanisms may yield novel insights into synucleinopathy pathogenesis, opening new avenues for targeted and holistic interventions.

The final challenge underscores the need for improved experimental and clinical models to investigate aSyn pathology. While transgenic animal models and patient-derived iPS cells have provided critical insights into aSyn aggregation and toxicity, they often fail to replicate key aspects of human pathology[273,282,364]. Novel approaches, such as CRISPR/Cas9-generated knock-in models and advanced 3D culture systems, could better capture disease-relevant processes and provide robust platforms for testing therapeutic interventions. Developing more physiologically relevant models will enhance the translational potential of preclinical research.

Addressing these challenges demands a multidisciplinary approach that combines cutting-edge technologies, robust experimental models, and clinical insights. Key priorities include unravelling the molecular triggers of aSyn aggregation, standardizing and validating biomarker detection assays, advancing therapeutic innovation through immunotherapies and gene-editing tools, and investigating the systemic interactions driving synucleinopathies. Collaborative efforts across scientific disciplines and integrating emerging technologies will be vital to overcoming these barriers.

By framing aSyn research within these critical challenges, the field is poised to make substantial progress in understanding and treating synucleinopathies. A comprehensive and strategic approach will drive progress in developing effective treatments, offering renewed hope for improved patient outcomes in these debilitating disorders.

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Abbreviations

The following abbreviations are used in this manuscript:

BBB	Blood-Brain Barrier
CMA	Chaperone-Mediated Autophagy
CNS	Central Nervous System
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Cerebrospinal Fluid
DLB	Dementia with Lewy Bodies
ER	Endoplasmic Reticulum
GBA1	Glucosylceramidase Beta 1
GCIs	Glial Cytoplasmic Inclusions
GWAS	Genome-Wide Association Study
IDP	Intrinsically Disordered Protein
LB	Lewy Body
LRRK2	Leucine-Rich Repeat Kinase 2
MAMs	Mitochondria-Associated Membranes
MRI	Magnetic Resonance Imaging
MSA	Multiple System Atrophy
NAC	Non-Amyloid Component
NMR	Nuclear Magnetic Resonance
PD	Parkinson's Disease
PET	Positron Emission Tomography
PMCA	Protein Misfolding Cyclic Amplification
PrPC	Cellular Prion Protein
RT-QuiC	Real-Time Quaking-Induced Conversion
SAA	Seed Amplification Assay
SN	Substantia Nigra
UPS	Ubiquitin-Proteasome System

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